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PATHOGENESIS OF THE RENAL INJURY IN CHOLINE DEFICIENCY: THE ROLE OF CATECHOLAMINES AND ACETYLCHOLINE

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Summary.—A disturbed renal circulation due to an imbalance between vasoconstrictor catecholamines and a vasodilator such as acetylcholine, caused by a decrease in acetylcholine, has been postulated as the basic mechanism of haemorrhagic degeneration of the kidneys in choline deficiency. To explore this hypothesis further a group of male weanling Wistar rats was fed on a choline-deficient diet for 10 days (Group CD). A control group was fed on the same basal diet supplemented with choline (Group CS). Food intake and body weights were registered. The kidneys of choline-supplemented and choline-deficient rats were studied grossly and histologically. The levels of catecholamines (noradrenaline and adrenaline) and acetylcholine were determined. Pathological changes of the kidneys were present in 30 out of 57 choline-deficient rats, permitting the separation of data obtained from deficient rats into those not associated with renal injury (CDa rats) and those associated with renal injury (CDb rats). A marked increase in the levels of renal catecholamines was observed in both CDa and CDb rats. On the other hand, the content of acetylcholine remained unchanged. It is noteworthy that the changes in tissue catecholamine levels occurred before there were changes in kidney weight and morphology. The findings support the concept that an imbalance between sympathetic and parasympathetic systems plays an important role in the pathogenesis of the renal injury of choline-deficient weanling rats, and that this imbalance would be the result of an excess of catecholamines in the kidneys.

SINCE THE DESCRIPTION by Griffith and Wade (1939) of haemorrhagic degeneration of the kidneys in choline deficiency, the study of this pathological change has attracted much attention. The specific mechanism, however, is still obscure. Decreased levels of phospholipids have been suggested as the cause of the renal lesions (Patterson and McHenry, 1944; Baxter and Goodman, 1955; Monserrat *et al.*, 1974), but it has been also postulated that an imbalance between vasoconstrictor catecholamines and a vasodilator such as acetylcholine, caused by a decrease in acetylcholine, could result in vasospasm, and subsequently ischaemia, necrosis, and haemorrhage (Wolbach and Bessey, 1942). A few reports have given presumptive evidence that a neurovascular disturbance occurs in acute choline deficiency (Dessau and Oleson, 1947; Baxter, 1952, 1953; Nagler, Baez and Levenson, 1969; Bruce, Weise and Carter, 1976). Nagler *et al.* (1968) have found, by direct bioassay, that the acetylcholine concentration in the kidneys of weanling rats fed a choline-deficient diet for 5 days was decreased 50–75% in comparison to control animals, thus speaking for the hypothesis that the basic mechanism underlying the renal injury of choline deficiency reflects an acute decrease in acetylcholine, which they believe would lead to abnormal vascular reactivity to catecholamines. No informa-

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tion exists in the literature, however, on the levels of both sympathetic and parasympathetic transmitters in the kidneys in choline deficiency. The current investigation was therefore undertaken to determine the levels of catecholamines and acetylcholine in the kidneys of normal and choline-deficient rats and, further, to correlate possible neurohormonal changes to alterations in renal morphology.

MATERIALS AND METHODS

Male Wistar albino rats of our outbred colony were used. The rats were weaned at 21 days and maintained for 3–5 days on commercial stock diet and water until they reached an average weight of 46 g. They were then divided at random into two groups: Group CD, consisting of 80 animals, was fed on a choline-deficient diet (choline-deficient group) *ad lib.*, and Group CS, consisting of 19 animals, was fed on a choline-supplemented diet (choline-supplemented group), also *ad lib.* Tapwater was freely available to both groups. The rats were individually housed in wire cages with raised bottoms and fed solid food in stainless-steel feeding dishes and water in drinking tubes. They were weighed on Days 1, 5 and 10 from the beginning of the experiment and their dietary consumption was recorded on Days 4, 7 and 10. The choline-deficient diet contained (g/100 g): vitamin-free casein—15·0; soybean oil—8·0; sucrose —70·7; salt mixture—5·0; vitamin mixture—1·0; cystein—0·3. The composition of the control diet was the same except for the supplementation of 200 mg of choline chloride per 100 g of diet. The vitamin mixture without choline was composed of (mg/100 g): retinol (100,000 u/g)—900; cholecalciferol (200,000 u/g)—50; α-tocopherol—500; ascorbic acid—4,500; inositol—500; menadione—225; *p*-aminobenzoic acid—500; niacin—450; thiamin—100; riboflavin—100; pyridoxine HCl—100; Ca panthotenate—300; biotin—2; folic acid—9; cyanocobalamin —0·135; dextrose to make 100 g. The salt mixture was prepared according to the Association of Official and Agricultural Chemists, containing the following amounts of salts (g/100): NaCl—13·945; KI—0·079; KH_2PO_4—38·9; $MgSO_4.7H_2O$ — 5·73; $CaCO_3$ — 38·14; $FeSO_4.7H_2O$ — 2·70; $MnSO_4.H_2O$ — 0·401; $ZnSO_4.7H_2O$ — 0·055; $CuSO_4.5H_2O$ — 0·048; $CoCl_2.6H_2O$—0·002. Both diets were practically isocaloric, providing 4·15 kcal/g.

After 10 days on test the surviving rats were killed under light ether anaesthesia by exsanguination from the abdominal aorta. Both kidneys were removed at once, examined macroscopically, and the left kidneys weighed.

The left kidneys from 8 choline-supplemented rats and from 35 choline-deficient rats were used for catecholamine analysis. Tissue catecholamines were separated and assayed according to the method of Anton and Sayre (1962, 1968). This method involves extraction of catecholamines from tissue homogenates with butanol, return of the amine to an aqueous phase, and the oxidation of the subsequent eluate to a fluorescent trihydroxyindole derivative in the presence of potassium ferricyanide and alkaline ascorbate. The fluorescence was read in an Aminco-Bowman spectrofluorometer. Readings were made at activation wave-lengths of 409 and 422 nm and at fluorescent wave-lengths of 519 and 529 nm for the assessment of noradrenaline and adrenaline, respectively. The catecholamine values are given in μg free base per g of wet tissue wt.

The left kidneys from 11 control rats and from 22 choline-deficient rats were used for acetylcholine bioassay. Acetylcholine was extracted by a technique based on the method of Rothshuh (1954). The organs are minced with scissors in their respective tubes and homogenized with 1·5 ml of 0·1N HCl (in an ice bath). After homogenization, 1·5 ml of 0·1N HCl is added and the tubes are immersed in boiling water for 5 min. The tubes are then left at room temperature for 10 min and centrifuged in a clinical centrifuge for 2 min. The supernatant fluid from each tube is decanted from the test tube and stored at −25°. The extracts were assayed biologically for acetylcholine by means of the isolated guinea-pig ileum suspended in Krebs–Ringer solution bubbled with air.

The right kidneys were incised and fixed by immersion in neutral 10% formalin solution, embedded in paraffin, and sections cut at 6 μm, stained with haematoxylin and eosin, and examined under the light microscope. Representative fragments from the median lobe of the liver were also processed for histological study.

Student's *t*-test was used for determination of statistical significance (Snedecor and Cochran, 1967). Differences between means which resulted in probability values (P) smaller than 0·05 were considered significant. Data are presented as mean ± standard error (mean ± s.e.).

RESULTS

Twenty-three animals of the choline-deficient group died spontaneously before the end of the experiment with renal haemorrhagic degeneration. This material was disregarded.

The mean daily consumption of solid food and calories, body weights and growth

TABLE I.—*Daily Solid and Caloric Intakes, Body Weights, and Growth Rates*

Groups	Body wt (g)		Growth rate	Food intake		Caloric intake
	Initial	Final	(g/day/rat)	g/day/rat	g/100 g	kcal/day/rat
CS	46·7 ± 1·19 (19)	83·2 ± 3·36 (19)	3·66 ± 0·24 (19)	9·09 ± 0·43 (19)	10·83 ± 0·24 (19)	37·72 ± 1·78 (19)
CD	46·8 ± 0·62 (80)	71·7 ± 1·35 (57)	2·48 ± 0·14 (57)	8·03 ± 0·19 (57)	11·21 ± 0·13 (57)	33·32 ± 0·79 (57)
P values	NS	< 0·0025	< 0·001	< 0·005	NS	< 0·005

The values represent the means ± s.e., with the number of animals in parentheses.
CS = choline-supplemented; CD = choline-deficient.

rates of both choline-supplemented (CS) and choline-deficient (CD) groups are given in Table I. The daily consumption of solid food for the control rats averaged 9·09 g/day/rat, which was higher than the average for the choline-deficient animals—8·03 g/day/rat. However, when food intake was related to animal body wt (g/100 g) average values were 10·8 and 11·2 for Groups CD and CS respectively. The total energy consumed by choline-supplemented rats (37·72 kcal/day/rat) was higher than the evaluated intake of calories of the deficient rats (33·32 kcal/day/rat). Retardation of body wt gain was experienced by Group CD as compared to the control. The growth rate of CD rats (2·48 g/day/rat) was significantly lower than the corresponding values of CS animals (3·66 g/day/rat). The control group increased in body wt by 78%, while choline-deficient rats increased only 53%.

On gross and histological studies renal changes were present in 30 out of 57 choline-deficient rats, permitting that the CD rats were separated into those without renal injury (now called CDa rats) and those with renal injury (now called CDb rats). The kidneys of choline-supplemented rats (CS) did not present any pathological

TABLE II.—*Left Kidney Weights, Left Kidney Weight:Body Weight Ratios, and Levels of Catecholamines and Acetylcholine*

	Groups			*P* values		
	CS	CDa	CDb	CS *vs* CDa	CS *vs* CDb	CDa *vs* CDb
Left kidney wt (g)	420·2 ± 18·7 (19)	410·2 ± 15·3 (27)	742·0 ± 36·4 (30)	NS	< 0·001	< 0·001
Kidney ratio (g/kg)	5·08 ± 0·14 (19)	5·52 ± 0·20 (27)	11·14 ± 0·69 (30)	< 0·05	< 0·001	< 0·001
Acetylcholine						
Concentration (μg/g kidney)	0·289 ± 0·056 (11)	0·317 ± 0·050 (7)	0·165 ± 0·031 (15)	NS	< 0·05	< 0·025
Total content (μg/kidney)	0·092 ± 0·017 (11)	0·090 ± 0·012 (7)	0·074 ± 0·012 (15)	NS	NS	NS
Noradrenaline						
Concentration (μg/g kidney)	0·289 ± 0·021 (8)	0·330 ± 0·017 (20)	0·275 ± 0·028 (15)	NS	NS	NS
Total content (μg/kidney)	0·109 ± 0·009 (8)	0·128 ± 0·04 (20)	0·200 ± 0·020 (15)	< 0·05	< 0·001	< 0·005
Adrenaline						
Concentration (μg/g kidney)	0·024 ± 0·005 (8)	0·041 ± 0·005 (20)	0·072 ± 0·007 (15)	< 0·025	< 0·001	< 0·005
Total content (μg/g kidney)	0·009 ± 0·002 (8)	0·016 ± 0·002 (20)	0·056 ± 0·007 (15)	< 0·025	< 0·001	< 0·001

The values represent the means ± s.e. with the number of animals in parentheses.
NS = not significant.
CS = choline-supplemented; CDa = choline-deficient without renal injury; CDb = choline-deficient with renal injury.

change on gross and light microscopic examination.

The left kidney weight: body weight ratios and the absolute weights of the left kidneys of CDb rats (11·14 ± 0·69 g/kg and 742·0 ± 36·4 mg respectively) were significantly higher than the corresponding values in CDa (5·52 ± 0·20 g/kg and 410·0 ± 15·3 mg) and CS rats (5·08 ± 0·14 g/kg and 420·0 ± 18·7 mg). The kidney ratio of CDa rats was significantly increased as compared to controls (Table II).

Table II shows the average values of concentration (expressed in μg/g wet tissue) and total content (expressed in μg/kidney) of catecholamines and acetylcholine in the left kidneys of choline-deficient (CDa and CDb) and choline-supplemented rats (CS). This is illustrated in Figs. 1 and 2.

The concentration of noradrenaline in the kidneys of CDa (0·330 ± 0·017 μg/g) and CDb rats (0·275 ± 0·028 μg/g) was found to be unaltered when compared to

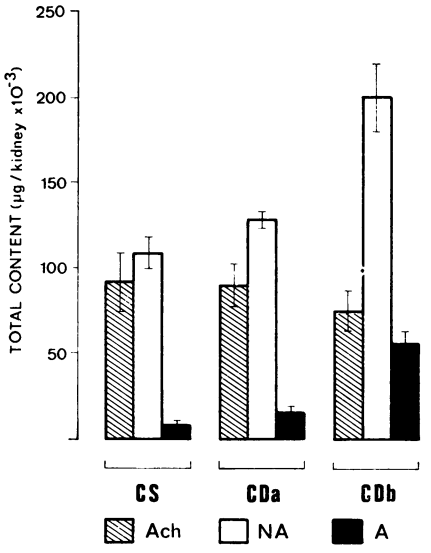

FIG. 2.—Total content of noradrenaline (NA), adrenaline (A), and acetylcholine (Ach) in the left kidneys of choline-supplemented rats (CS), choline-deficient rats without renal injury (CDa), and choline-deficient rats with renal injury (CDb). The vertical bars represent the s.e.m.

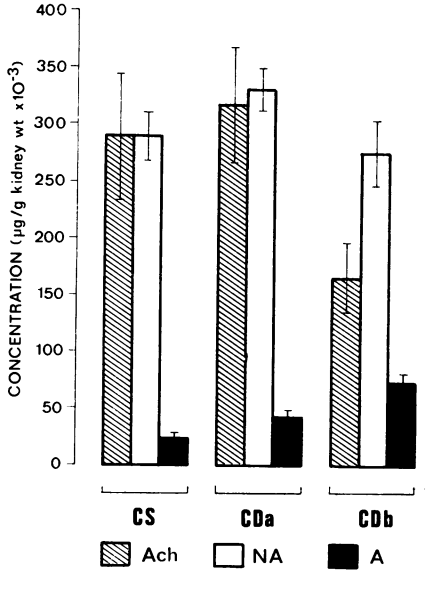

FIG. 1.—Concentrations of noradrenaline (NA), adrenaline (A), and acetylcholine (Ach) in the left kidneys of choline-supplemented rats (CS), choline-deficient rats without renal injury (CDa), and choline-deficient rats with renal injury (CDb). The vertical bars represent the s.e.m.

CS animals (0·289 ± 0·021 μg/g). On the other hand, the total content of noradrenaline in the kidneys of CDa (0·128 ± 0·004 μg/kidney) and CDb rats (0·200 ± 0·020 μg/kidney) was significantly increased in comparison to CS rats (0·109 ± 0·009 μg/kidney). Furthermore, the total content of noradrenaline of CDb rats was significantly higher than the corresponding values in CDa rats.

In the kidneys of CDb rats the total content (0·056 ± 0·007 μg/kidney) as well as the relative amount (0·072 ± 0·007 μg/g) of adrenaline were markedly higher than the corresponding values of CDa (0·016 ± 0·002 μg/kidney and 0·041 ± 0·005 μg/g) and CS rats (0·009 ± 0·002 μg/kidney and 0·024 ± 0·005 μg/g). Besides, the total content and also the concentration of adrenaline in CDa rats were significantly higher than those in CS animals.

While the values of concentration and total content of acetylcholine in the kidneys of CDa rats (0·317 ± 0·050 μg/g and 0·090 ± 0·012 μg/kidney) did not differ

from those of CS rats ($0{\cdot}289 \pm 0{\cdot}056$ μg/g and $0{\cdot}092 \pm 0{\cdot}017$ μg/kidney), the concentration of acetylcholine in CDb rats ($0{\cdot}165 \pm 0{\cdot}031$ μg/g) was significantly lower than those of CDa and CS rats. However, no differences were found between the values of total content of acetylcholine in CDb rats ($0{\cdot}074 \pm 0{\cdot}012$ μg/kidney) and those of CDa and CS rats.

Macroscopically the livers of choline-deficient rats were yellowish in contrast with the reddish-brown appearance of the livers of control animals. Liver sections from deficient rats showed marked hepatocytic vacuolization involving the entire lobule, but predominantly the centrilobular region. The control group had normal hepatic structure.

DISCUSSION

Rats given a choline-deficient diet showed restriction of body wt gain. Further, the weights of damaged kidneys from CDb rats (expressed in absolute values or as percentages of body wts) were higher than the wts of kidneys from CDa and CS rats. On the other hand the absolute weights of kidneys from CDa rats did not differ from those of CS animals, while the kidney ratio of CDa rats were significantly increased in comparison to controls. This difference can be attributed to the deficient gain in weight of rats fed a choline-deficient diet. The increased weight of damaged kidneys, which is mainly due to haemorrhage and swelling, has been invariably found in previous investigations (Griffith and Wade, 1939; Györgi and Goldblatt, 1940; Christensen, 1942; Wolbach and Bessey, 1942; Dessau and Oleson, 1947; Monserrat, Porta and Hartroft, 1968).

A disturbed renal circulation due to an imbalance between sympathetic and parasympathetic systems has been postulated as the basic mechanism of the renal injury of choline deficiency. This imbalance, caused by an insufficiency of acetylcholine, would result in vasospasm, and thus ischaemia, necrosis and haemorrhage. Since the first report of Wolbach and Bessey (1942) suggesting a neurovascular mechanism in the causation of the renal lesions in choline deficiency, several papers exploring this hypothesis have appeared in the literature.

Dessau and Oleson (1947) have shown that the renal damage due to choline deficiency is considerably reduced by prior renal decapsulation, so this effect would appear to be a result of disturbing renal nerves. These findings were not confirmed by Hartroft (1948), but a similar observation was described by Baxter (1952). In the same brief communication, Dessau and Oleson have also reported that almost 50% of choline-deficient rats failed to show renal lesions when they were treated with atropine daily from the day they were started on the choline-deficient diet. These results, however, could not be confirmed by Hartroft (1948) and Baxter (1953), who were unable to protect rats from acute choline deficiency nephropathy with atropine.

Baxter (1953) has found that while dibenamine, a blocker of α-adrenergic receptors, afforded protection against the renal injury of choline deficiency, the use of dibenzyline, another adrenergic blocking agent, was ineffective. However, because of the severe local irritation and induration of tissues caused by dibenamine, and the negative results with dibenzyline, he interpreted the protective effects of dibenamine as resulting from an "alarm reaction" and not from an adrenergic blocking effect.

Nagler *et al.* (1968) have related that in choline deficiency there is a fall in renal acetylcholine concentration, suggesting that a hypersensitivity to catecholamines results from this insufficiency, with subsequent vasospasm and ischaemic damage. Nagler, Baez and Levenson (1969) have since reported an increased sensitivity to adrenaline of the mesoappendiceal circulation of rats fed a choline-deficient diet in comparison to choline-supplemented rats.

Kratzing, Wetzig and Boland (1970) have observed a marked lowering of the increased blood pressure of choline defi-

cient rats given phenoxybenzamine, an adrenergic blocking agent. Similar results were reported by Kratzing, Wetzig and Ellway (1970) using α-methyldopa, a drug that depletes the tissue stores of noradrenaline. These findings suggest that adrenergic mechanisms control choline-induced hypertension, which could be also implicated in the development of renal pathological changes.

More recently, Bruce, Weise and Carter (1976) were able to demonstrate that choline-deficient rats had a higher excretion of urinary catecholamines than control animals supplemented with choline. Further, deficient rats injected with reserpine, which depletes the stores of noradrenaline in both central and peripheral nervous systems, had significantly less renal necrosis and excreted less catecholamines than choline-deficient rats.

The results of the present experiment clearly show that the choline-deficient diet induced a markedly significant increase in the values of total content of noradrenaline and adrenaline and concentration of adrenaline in the kidneys of weanling rats. However, the values of concentration of noradrenaline in the kidneys of choline-deficient rats were not different from those of control rats. These findings are supported by those previously reported by Bruce *et al.* (1976). Moreover, the separation of data obtained from choline-deficient rats into those not associated with renal injury (CDa) and those associated with renal injury (CDb) permitted the observation that the content of renal catecholamines of CDa rats was significantly higher than that of choline-supplemented rats. In other words, the changes in tissue catecholamines occurred before there were changes in kidney weight and morphology. This is fundamental because it could be attributed to an increased activity of the sympathetic system, due to renal haemorrhage, the increased levels of catecholamines in the kidneys of choline-deficient rats. On the other hand, the total content of acetylcholine in the kidneys of choline-deficient rats (with or without renal injury) was not different than the content of the neurotransmitter in the kidneys of control rats given the supplement of choline. However, the concentration of acetylcholine in deficient rats with renal injury was significantly lower than those of deficient rats without renal injury and controls, but of course these differences can be attributed to the markedly increased renal weights of rats with renal lesions. In contrast with these results, Nagler *et al.* (1968) have shown a reduction in the concentration of acetylcholine in brains, kidneys, and intestines of rats deprived of choline for 6 days after weaning. However, our findings are in agreement with those of Haubrich *et al.* (1976) showing that choline deficiency had no effect on the concentration of acetylcholine in the brain, duodenum, heart, kidney, or stomach of rats in comparison to control animals receiving choline supplements.

In summary, the present findings support the concept that an imbalance between sympathetic and parasympathetic systems plays an important role in the pathogenesis of the renal injury of choline-deficient weanling rats; and this imbalance would seem to be the result of an excess of catecholamines in the kidneys.

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